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Evaluation of the antimicrobial activities of different solvent extracts from the bark of *Acacia Stenophylla* A. CUNN. EX. BENTH.

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Key words: Antibacterial, Antifungal, *Acacia stenophylla*, Well diffusion.

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Abstract

The present study was conducted to evaluate the *in-vitro* antimicrobial activities of crude extracts derived from the bark of *Acacia stenophylla* by different organic solvents such as N-Hexane, Chloroform, Ethyl acetate, and N-Butanol. These solvent extracted samples were applied against three Gram-positive bacteria including *Bacillus atrophaus, Bacillus subtilis,* and *Staphylococcus aureus*, as well as four Gram-negative bacteria that comprised *Klebsiella pneumonia*, *Pseudomonas aeruginosa, Salmonella typhi,* and *Escherichia coli*. Additionally, the antimicrobial activities of these samples were also tested against one fungal strain i.e. *Candida albicans*. The antimicrobial potentials were measured by using the Well diffusion method in three different doses of 1, 2, and 3 mg, respectively. The antibiotic ciprofloxacin, anti-fungal clotrimazole, and DMSO were used as positive and negative control. Our results suggest that *Salmonella typhi* was a highly sensitive bacterial strain followed by *Staphylococcus aureus* showing 87.34% and 72.41% ZI, respectively. Similarly, *Bacillus atrophaus* was found the most resistant bacterium tested. N-hexane was highly potent against *Salmonella typhi* that indicates that biologically active substances against *S.typhi* are non-polar. N-Hexane fraction showed maximum inhibitory potential against gram-positive bacteria as compared to other solvent fractions while crude methanol demonstrated maximum anti-microbial strength against Gram-positive, Gram-negative bacteria as well as fungal strain. This specifies the broad-spectrum antibacterial and antifungal potential of the crude methanol. Our study verified that crude methanol and its different solvent fractions extracted from the bark of *A. stenophylla* have promising antimicrobials that can be used against various bacterial and fungal diseases. Furthermore, a study is needed for the optimization of antimicrobials of the plant for commercial utilization.

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Introduction

The remedial use of plants in traditional medical systems has been in practice since ancient times, and globally about 60% of the people are still linked to the use of traditional medicine (Nielsen *et al.,* 2012). In Pakistan too, the huge population depends on the traditional plants for the treatment of both trivial and in some cases major ailments (Shinwari, 2010). About 720 out of 6000 higher plant species in Pakistan are believed to be medicinally important. The efficacy of medicinal plants is due to the combination of secondary metabolites present in them (Briskin, 2000; Khan *et al*., 2017b).

Globally, the genus *Acacia* encompasses approximately 1350 species. Of them, 960 species are native to Australia (Ogunbinu *et al*., 2010). The genus *Acacia* provides a wide range of therapeutic applications. For example, different species of the genus *Acacia* have been explored for various pharmacological activities in different regions of the world. These include, but are not limited to, antimicrobial, antifungal, antiviral, analgesic, anthelmintic, antioxidant, antipyretic, antispasmodic, anti-infertility, antidiarrheal, antidiabetic, antihypertensive, anti-Alzheimer's, and anti-malarial activities (Subhan *et al*., 2018). The traditional use of *Acacia* in Africa and some other Asian countries like Pakistan, Sri Lanka, India, Bangladesh accounted for almost 80 percent (Subhan, 2016). Keeping in view the massive medicinal value and pharmacological activities of the genus *Acacia,* the current study was conducted to evaluate, for the first time, the antibacterial and antifungal activities of *Acacia stenophylla* in different solvent extracts/fractions.

Material and methods

Plant material

The bark of *Acacia stenophylla* was collected in August 2017 from Pakistan Forest Institute University of Peshawar. Following the identification process the specimen was deposited in the herbarium (ICP). They were dried in shade at the botanical garden of Islamia College Peshawar. It took about three weeks to make it completely dry, then the dried plant bark was grounded which yielded 7.5 Kilogram (Kg) powder.

Extraction process

The extraction process was carried out in steel drums. Initially, approximately 32 liters of commercial-grade methanol (Musa Je Karachi market, Peshawar) was added to the drums containing the powder. The powder was completely submerged in methanol for about one month while regular stirring was carried out every 3 days. After one month of soaking the powder in methanol, the filtration process was started. The methanolic extract was first filtered through a velvet cloth followed by another filtration through Watt's Man filter paper. The filtrate was then concentrated using a rotary evaporator machine. The rotary evaporator was used to separate the methanol from the plant extracts. The temperature of the rotary evaporator was set at 50 °C and 60 rpm. After using a rotary evaporator, further concentration was carried out using a water bath at a temperature of 50 °C. It took about two weeks to make the filtrated plant extract completely concentrated.

Fractionation

The fractionation was done by escalating order of polarity i.e. N-Hexane, Chloroform, Ethyl acetate, N-butanol, Methanol, and Aqueous Fraction. The methanolic extract of *Acacia stenophylla* was mixed in distilled water, which was dissolved in it by stirring and heating to form an aqueous solution, the aqueous solution was poured in two separating funnel of volume 1000ml. Then N-hexane was added into both funnels and the funnels were thoroughly shacked to attain the equilibrium, and both the funnels were fixed in the stands. After a short period, two layers were formed inside the funnels, on the top was the N-hexane layer and at the bottom was the aqueous layer. Then, a small flask was placed underneath each separating funnel and the aqueous layer was run down the tap while the N-hexane layer was collected from the top opening of the funnel. This procedure was replicated three times to get maximum N-hexane extract. Finally, a rotary evaporator was used to concentrate the hexane fraction.

Then chloroform solvent was added to the watery solution taken in two separating funnels, two layers

were formed, this time the chloroform layer was at the bottom while the aqueous layer was on the top. The chloroform was collected in flasks placed underneath the separating funnels while the aqueous solution was taken from the upper opening of the funnels. This process was also repeated three times to get maximum chloroform extract.

The aqueous solution was taking into two separating funnels and ethyl acetate was added, having a greater density than ethyl acetate the water form layer at the bottom of the funnel while ethyl acetate made the top layer.

The aqueous layer was collected in a flask placed underneath the separating funnel while the ethyl acetate was collected from the upper opening and this practice was carried out four-folds. At last, N-butanol was added to the aqueous layer and the above-mentioned procedure was repeated three-folds. N-butanol being having a higher boiling point i.e. about 117 °C took much time while concentrating in a rotary evaporator. While concentrating the N-Butanol fraction the temperature was set at 90 °C and 70 rpm.

Well Diffusion susceptibility assay

Two different media were used i.e. nutrient broth for incubation and standardization and nutrient agar was used for culturing and growth of microbial strains. Antimicrobial activity of different fractions was carried out by Well Diffusion Method as described by (Atta-ur-Rahman and Thomson, 1999; Azam *et al.*, 2016). Three wells of equal size were formed through sterilized borer in all nutrient agar media Petri plates and they were then labeled properly. The plates were then inoculated with 50ul of specific microbial inoculum (24 hours culture). Each plant extract was applied in three different concentrations such as 1mg, 2mg, and 3mg in the volumes of 6ul, 12ul, and 18ul respectively into wells. Antibiotics were used in separate plates as a positive control. All the inoculated plates were incubated for 24 hours at 37 °C and the inhibitory potential (zone of inhibition) was measured in mm around the wells in each plate by the next day.

Positive control

Ciprofloxacin was used as a positive control for both Gram-positive and Gram-negative bacteria in the concentration of 50µg/12µl and clotrimazol was used against the fungal strain.

Statistical analysis

The data represent the mean value and standard deviation of three replicates. SPSS software version 22 was applied for the statistical analysis of the data.

Results

The data in Fig. 1 showed the antibacterial activity of five different solvents extracts against *Bacillus atrophaeus*. The data assessment illustrated that all fractions had ZI above 50% at all concentrations except chloroform. Maximum ZI was shown by methanolic extract which was 57.12% at a concentration of 3mg/well, followed by ethyl acetate and methanolic fractions (54.75%) at concentrations of 3mg and 2mg respectively. The data in Fig. 2 revealed that both chloroform and methanol plant extract had excellent ZI (i.e. above 60 %) against *Bacillus subtilis* at all concentrations i.e. at 1mg/6µl, 2ml/12µl, and 3mg/18µl as compared to other solvent fractions like Ethyl acetate, N-Butanol, and N-Hexane. Similarly, N-Butanol and ethyl acetate extracted samples showed moderate antibacterial activity. The highest ZI of 71.42% was measured for methanolic extract at higher concentrations, followed by chloroform (67.85%). Similarly, the lowest ZI was shown by N-Hexane which was 39.28% at concentrations of 1 and 2mg. Fig. 3 depicted the antibacterial potential of various plant extracts against *Escherichia coli*. All the solvent extracted samples demonstrated ZI above 50%, however, maximum bacterial growth inhibition was observed for Methanol and N-Butanol extracted samples.

The remaining samples showed almost similar activity (round about 50%). The results declared that the highest antibacterial activity was recorded for methanol extracted sample at a concentration of 3mg which was 68.23%, followed by an N-Butanol fraction (65.90%) at the same concentration.

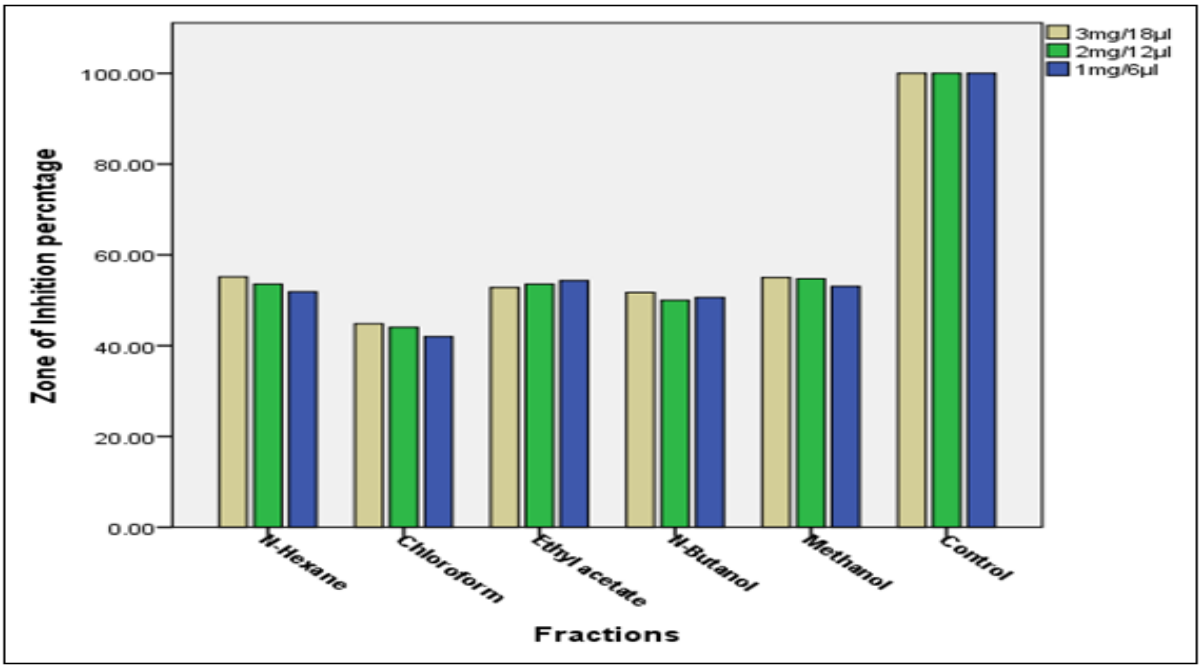

Fig. 1. Antibacterial activity of n-hexane, Chloroform, Ethyl acetate, n-butanol, and Methanol fractions from *Acacia stenophylla* against *Bacillus atrophaeus* by Well Diffusion Method.

Different solvent extracted samples of *Acacia stenophylla* were subjected to inhibit the growth of *Pseudomonas aeurogenosa* in Fig. 4. All extracted samples inhibited the growth except N-Butanol (showed no activity at all concentrations). The results declared that all fractions revealed good inhibitory potential. The highest ZI (i.e. 64.37%) was shown both by Chloroform and Methanol extracted samples at higher concentrations of 3 mg. Lowest ZI was recorded for N-Hexane which was 40.24% at 1 mg. Similarly, Ethyl acetate inhibited the growth of tested bacterium by 62.06%, 58.62%, and 57.48% at concentrations of 3mg, 2mg, and 1mg respectively.

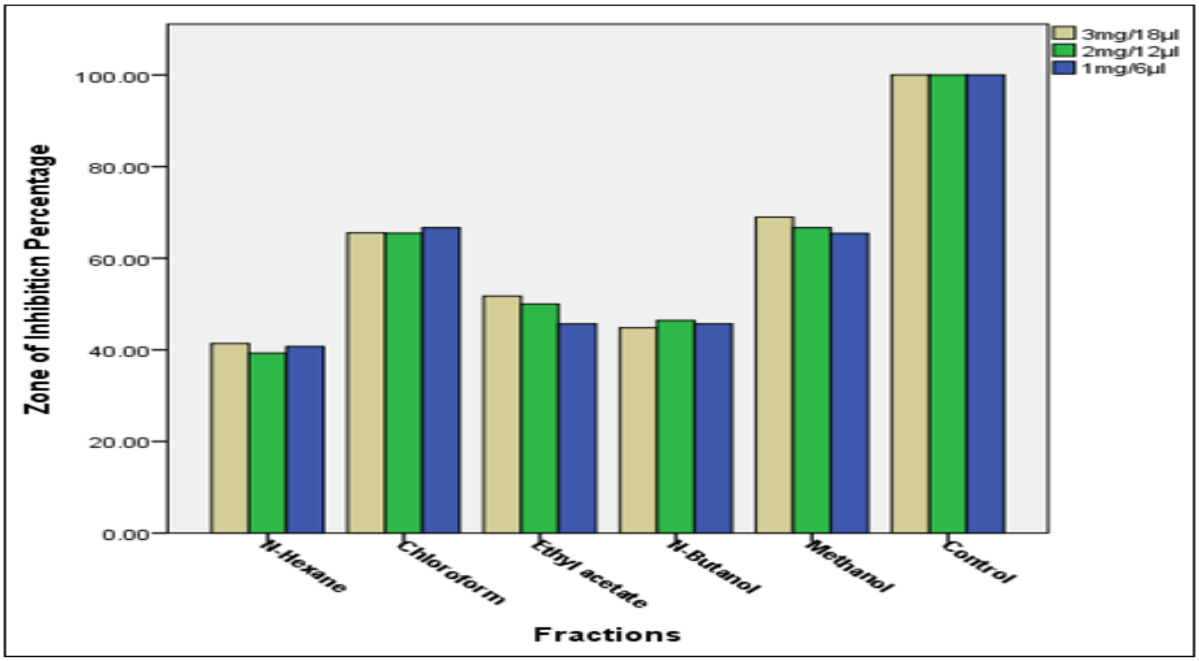

Fig. 2. Antibacterial activity of n-hexane, Chloroform, Ethyl acetate, n-butanol, and Methanol fractions from *Acacia stenophylla* against *Bacillus subtilis* by Well Diffusion Method.

Our results in Fig. 5 disclosed the inhibitory potential of *Acacia stenophylla* against *Klebsiella pneumonia*. Chloroform extracted samples showed poor activity i.e. less than 40%. The remaining fractions possessed moderate activity. N-Butanol and N-Hexane samples inhibited the growth of *Klebsiella pneumonia* by 56.95% and 55.80% respectively at a concentration of 3mg. Ethyl acetate and methanol extracted samples showed concentration-dependent ZI. With increasing concentration of the tested samples, more inhibition was observed.

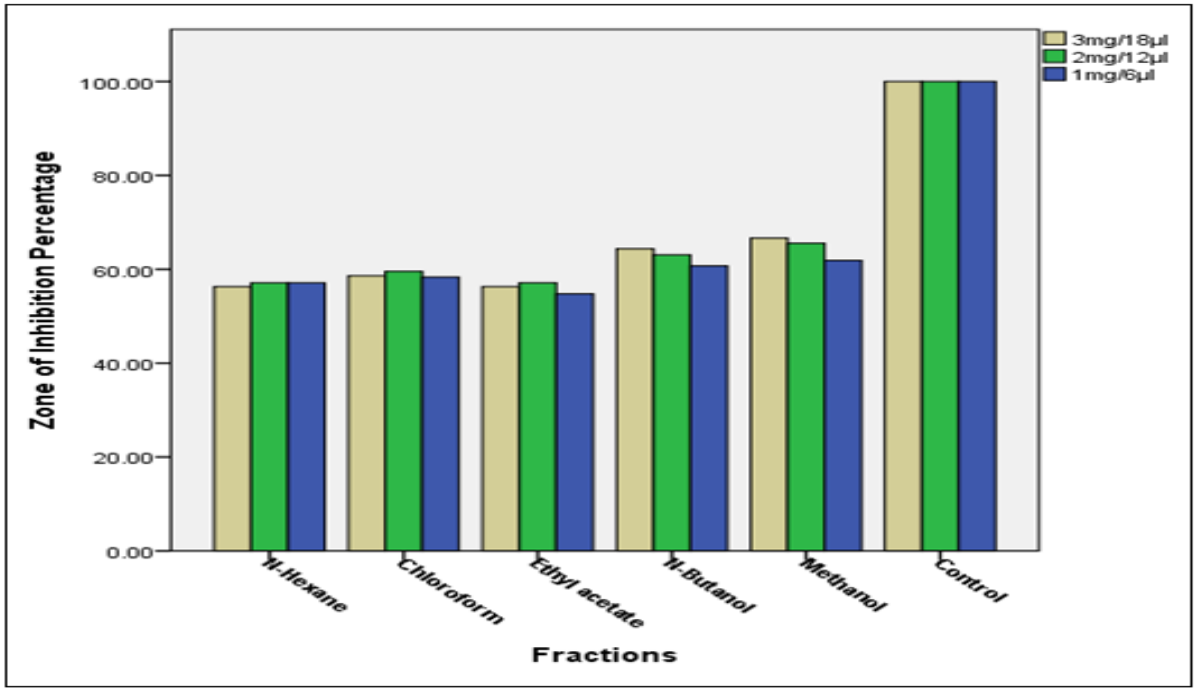

Fig. 3. Antibacterial activity of n-hexane, Chloroform, Ethyl acetate, n-butanol, and Methanol fractions from *Acacia stenophylla* against *Escherichia coli* by Well Diffusion Method.

The antibacterial potential of *Acacia stenophylla* against *Staphylococcus aureus* was found to be concentration-dependent. As shown in Fig. 6, all the tested samples reduced the growth of the *Staphylococcus aureus* more prominently at high concentrations. Ethyl acetate fraction showed the maximum potential of 72.41% at 3mg, followed by N-Butanol (66.65%) at 1 and 2mg. Likewise; the minimum activity of 35.62% was recorded for N-Hexane at a concentration of 1mg. The results further elaborated that Chloroform extracted samples reduced the growth by 51.72% at 1 and 2mg and 56.31% at 3mg concentration. A similar pattern of inhibition was also observed for methanol fraction.

The most significant growth inhibitory activity was shown by N-Hexane fraction against *Salmonella typhi* in Fig. 7. The N-Hexane reduced the growth of the bacterium by 87.34% at a higher concentration of 3mg. Similarly, concentrations of 2mg and 1mg showed 83.54% and 73.40% ZI respectively. The remaining fractions showed activity of up to 60%. Ethyl acetate and Methanol extracted samples inhibited the growth by 62.02% and 60.75% at 3mg respectively. Similarly, Chloroform and N-Butanol possessed almost the same inhibitory potential.

The antifungal activity of *Acacia stenophylla* against *Candida albicans* has been depicted in Fig. 8. Various solvent extracted samples were screened for reducing the growth of the tested fungal strain. The results declared that methanol and N-Butanol fractions showed a comparatively better inhibitory effect.

The maximum inhibition was measured for methanol which was 68.75% at 3mg, followed by N-Butanol (65.63 %) at higher concentration. The N-Hexane possessed poor inhibitory activity.

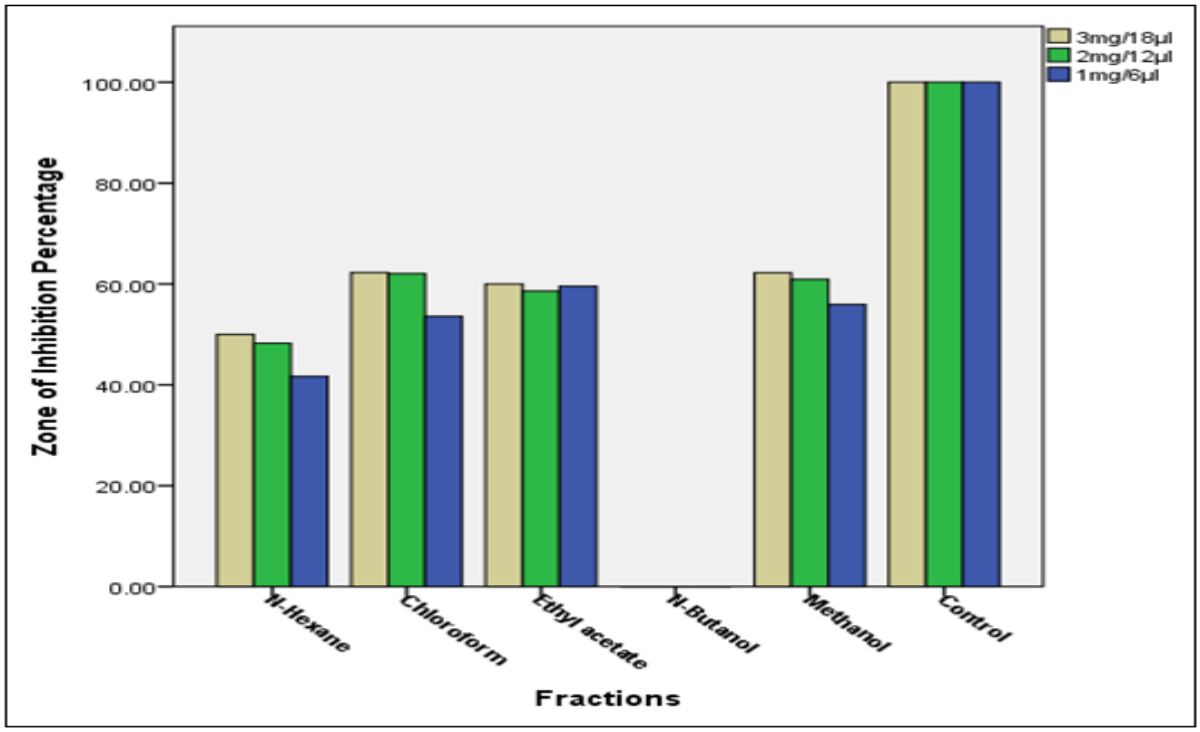

Fig. 4. Antibacterial activity of n-hexane, Chloroform, Ethyl acetate, n-butanol, and Methanol fractions from *Acacia stenophylla* against *Pseudomonas aeruginosa* by Well Diffusion Method.

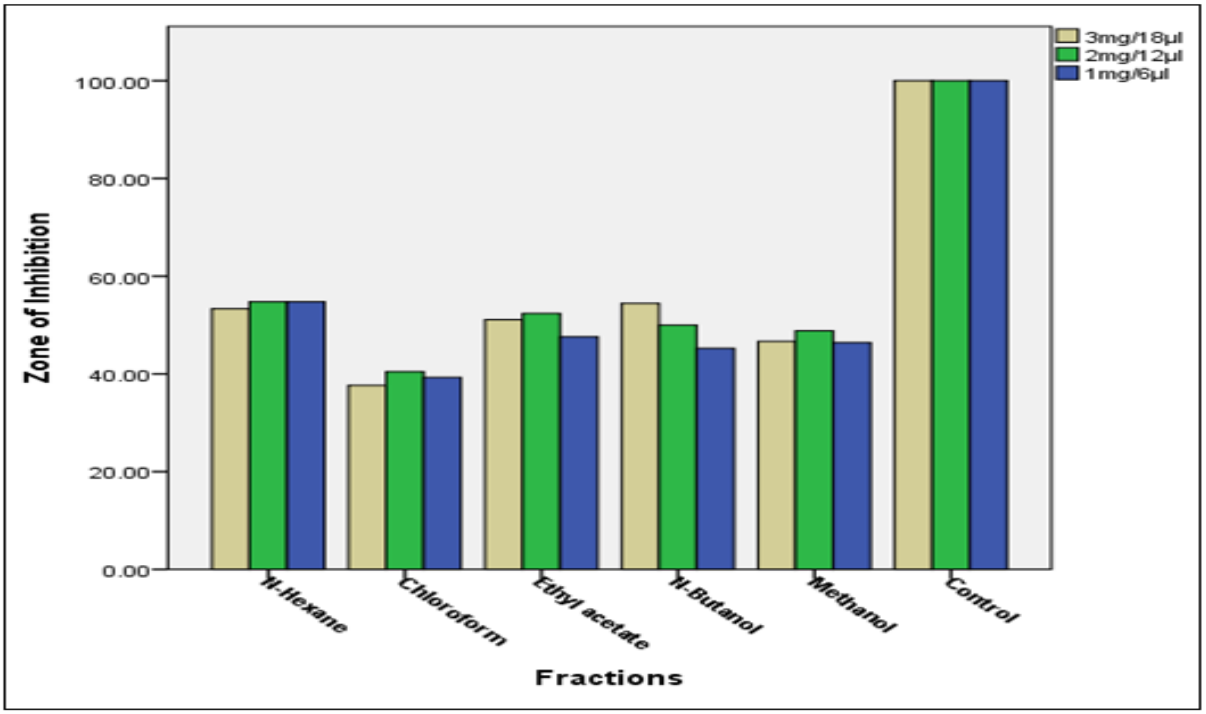

Fig. 5. Antibacterial activity of n-hexane, Chloroform, Ethyl acetate, n-butanol, and Methanol fractions from *Acacia stenophylla* against *Klebsiella pneumonia* by Well Diffusion Method.

Discussion

Our results suggest significant alleviation in the growth of all tested microorganisms by all extracted plant samples. Irrespective of the microbial strains, all tested bacterial and fungal strains demonstrated considerable sensitivity to all extracted plant samples at all three doses. This implies the broad-spectrum antibiotic potential of the crude methanol and its

different solvent fractions of the plant. A therapeutic plant that inhibits the growth of bacteria at a range that exceeds 6 mm is deemed to have antimicrobial potential (Wang *et al.*, 2008). The result revealed that reduction in the growth of *Bacillus atrophaeus* occurred at all types of solvent extracted plant fractions at each concentration, however, maximum and equal inhibition in growth occurred at a high dose of crude Methanol and N-Hexane fractions (57.12 %), Ethyl acetate was second-best in controlling *B. atrophaeus*. However, minimum activity (40.46 %) was shown towards Chloroform fraction at a lower concentration. Similar observations were given by (Khan *et al.*, 2017a) for crude methanol and its different fractions from the stem of *Ephedra geradiana*. Additionally, *Bacillus subtilis* showed the highest degree of sensitivity towards crude Methanol and its Chloroform fraction at different concentrations, and its sensitivity augmented with raise in concentration (i.e. from 63.10 % to 71.42 % and from 64.28 % to 67.85 %) respectively which indicated that active anti-microbial constituents against *Bacillus subtilis* increase at high dose, the result was in line with (Bakht *et al.*, 2013; Onuegbu *et al.*, 2019). *Bacillus subtilis* showed the least sensitivity towards N-Hexane at each concentration as compared to other fractions; similar results were observed by (Bakht *et al.*, 2014). Conversely, *Klebsiella pneumonia* showed the highest degree of sensitivity towards N-Hexane at each concentration as compared to control and other fractions, the result was in concordance with (Ahmad *et al.*, 2011; Rahman and Rashid, 2008; Ullah *et al.*, 2014).

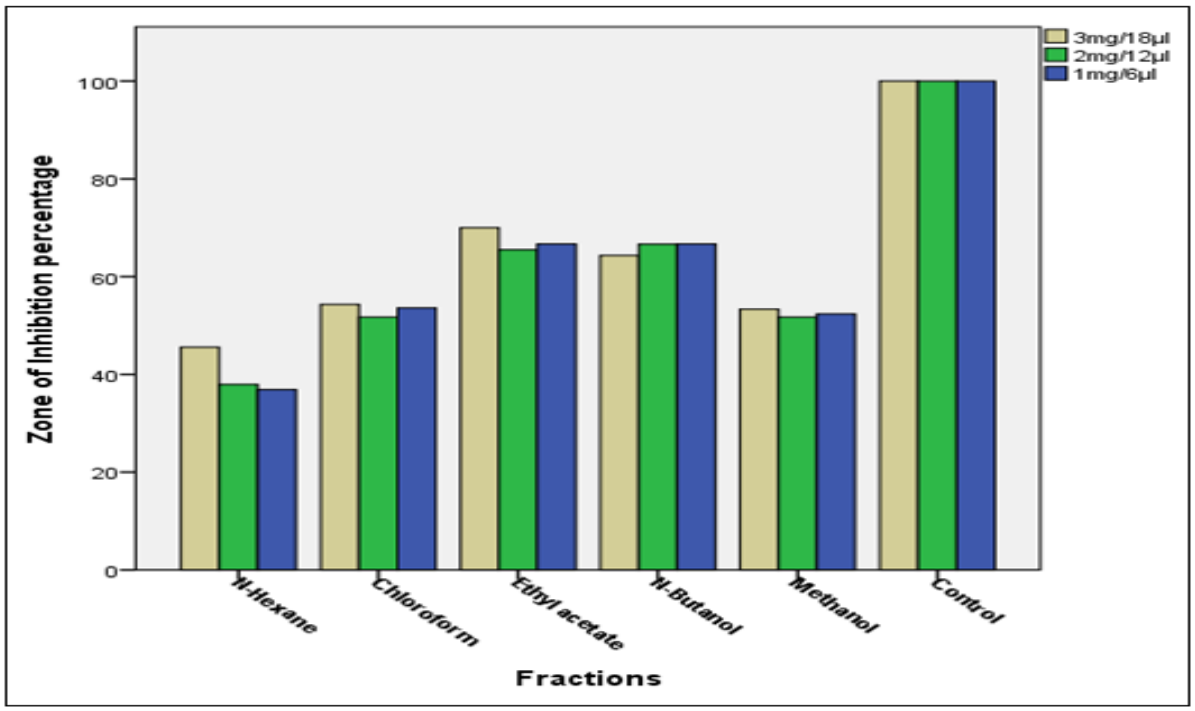

Fig. 6. Antibacterial activity of n-hexane, Chloroform, Ethyl acetate, n-butanol, and Methanol fractions from *Acacia stenophylla* against *Staphylococcus aureus* by Well Diffusion Method.

The results proved that *Escherichia coli* was highly vulnerable toward crude methanol (68.23 %) and N-Butanol (65.90 %) extracts at high dose and its sensitivity increased in a dose-dependent manner, alike it showed moderate susceptibility towards Chloroform, Ethyl acetate, and N-Hexane at all three concentrations. The result was on par with (Fazal *et al.*, 2012; Khan *et al.*, 2017b). The analysis of the result proved that *Pseudomonas aeruginosa* showed resistance towards N-Butanol at each concentration and higher sensitivity towards Chloroform at high dose, followed by Ethyl acetate with almost the same ZI, this result was in line with the results of (Lim *et al.*, 2009) but contradictory to (Khan *et al.*, 2017b)

who observed maximum sensitivity of *Pseudomonas aeruginosa* towards N-Hexane instead of Chloroform. The data indicated that Ethyl acetate has the highest ZI against *Staphylococcus aureus* at each concentration whereas N-Hexane was least effective against *Staphylococcus aureus*.

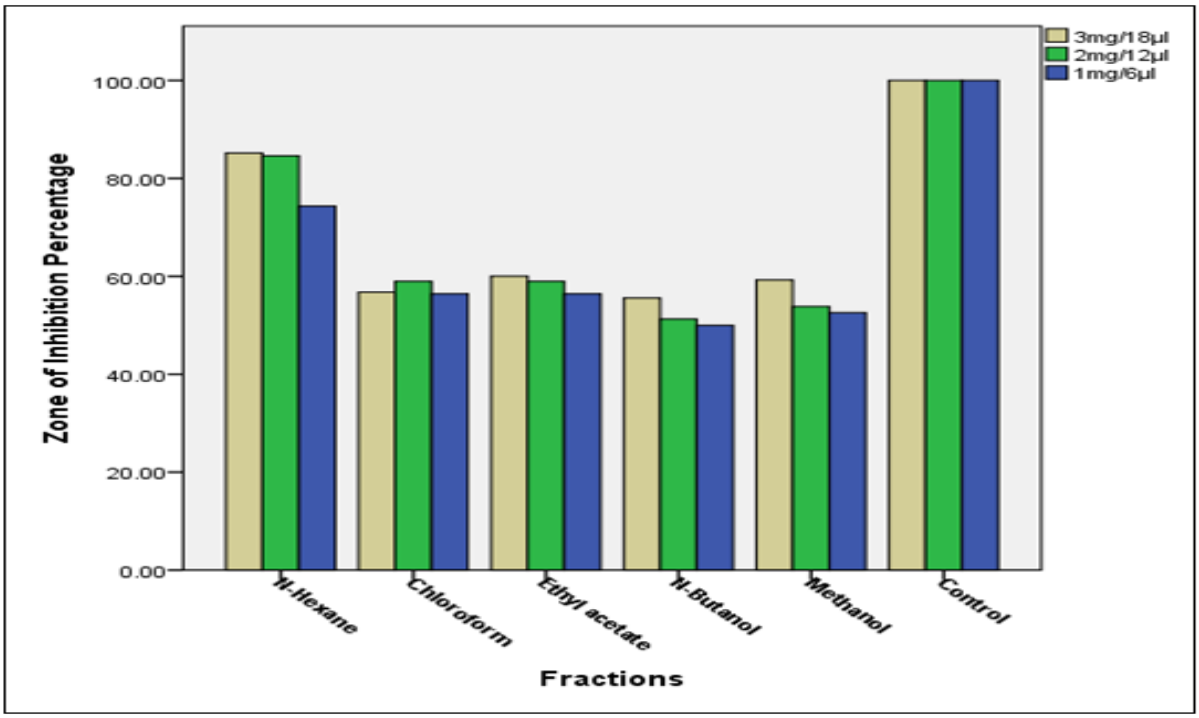

Fig. 7. Antibacterial activity of n-hexane, Chloroform, Ethyl acetate, n-butanol, and Methanol fractions from *Acacia stenophylla* against *Salmonella typhi* by Well Diffusion Method.

The result was in agreement with (Njeru *et al.* ,2015) but did not coincide with (Khan *et al.*, 2013; Berfad and Alnour, 2014) who observed the least sensitivity of *Staphylococcus aureus* towards Ethyl acetate. The high effectiveness of Ethyl acetate might be due to the presence of a high concentration of phenolic and terpenoid compounds because *Staphylococcus aureus* is particularly more sensitive to phenolic and terpenoid compounds (Cowan, 1999; Subedi *et al.*, 2012).

The data also revealed that N-Hexane was more effective against *Salmonella typhi* at each concentration and the growth of the test microorganism was reduced in a concentration-dependent manner (i.e. 73.4 to 87.34 %). The result was endorsed by (Nabèrè *et al.*, 2013) who observed the highest ZI for the N-Hexane fraction of *Nelsonia canescene*. Similarly, all other solvent fractions including crude Methanol indicated moderate activity against *Salmonella typhi*. Our results specified that crude Methanolic extract was highly effective in inhibiting the growth of fungus *Candida albicans* with 68.75 % ZI, followed by ethyl acetate and N-Butanol plant fraction having uniformly 65.63 % ZI for each. Comparable observations were recorded by (Khan and Bakht, 2016).

The anti-microbial potential of the crude Methanol and its different fractions are attributed to the presence of various phyto-active constituents like alkaloids, steroids, terpenes, sterols, flavonoids, tannins. Saponins, and anthraquinones, and other secondary metabolites as already reported in the methanolic extract of the *Acacia stenophylla* plant by (Iqbal, 2015).

The antimicrobial potential of these secondary metabolites is mentioned by (Akinsulire *et al.*, 2007). The fractionation of the crude metabolic extract raised the anti-microbial potential of different solvent fractions against various gram-positive and gram-

negative bacteria as can be seen in the case of N-Hexane which has 87.34 % inhibitory potential against *S. typhi* while crude Methanol has a minimum 51.91 % potential against the tested bacteria. Likewise; Ethyl acetate has the maximum activity of 72.41 % against *S. aureus* while crude methanol has only 50.57 % activity against the *S. aureus*.

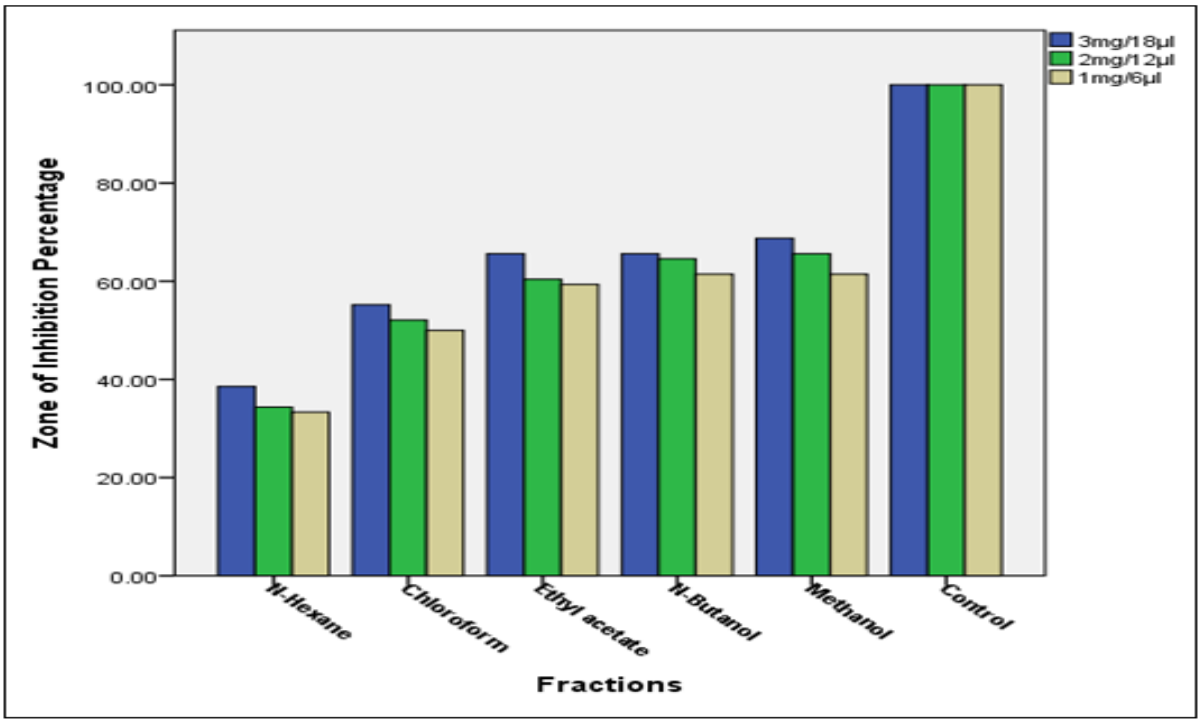

Fig. 8. Antibacterial activity of n-hexane, Chloroform, Ethyl acetate, n-butanol, and Methanol fractions from *Acacia stenophylla* against *Candida albicans* by Well Diffusion Method.

Conclusion

In summary, our results suggest that crude methanol was only potent in inhibiting the growth of gram-positive *Bacillus atrophaeus, Bacillus subtilis* and gram-negative *Escherichia coli,* while other solvent fractions like N-hexane, Ethyl acetate and N-butanol were more effective than its crude methanol in controlling the growth of other bacterial and fungal strains used.

The variation in the effectiveness of the extracts could be attributed to the types and concentration of the biologically active constituents of the extract used. Finally, the inherent susceptibility of each bacterial or fungal strain could also explain the observed variation in the efficacy of different solvent extracts used in this study. The study concluded that different solvent fractions extracted from the bark of *A.stenophylla* possess varying antimicrobials that can be optimized in the future for commercial utilization.

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